AMENDMENTS TO THE SPECIFICATION

Please substitute the below amended paragraph for the paragraph on page 1, lines 5-9.

This application is a Division of 09/633,739 filed 08-07-2000 now patented as 6,635,447; which is a Division of 08/456,108 filed 05-30-1995 now patented as 6,100,054; which is a Division of 08/145,681 filed 10-28-1993 now patented as 5,571,691; which is a Continuation-in-part of 07/967,947 filed 10-27-1992 now Abandoned; which is a Continuation-in-part of 07/873,304 filed 04-24-1992 now Abandoned; which is a Continuation of 07/348,270 filed 05-05-1989 now Abandoned.

Please substitute the below amended paragraph for the paragraph on page 6, lines 6-8.

Fig. 2 is the cDNA sequence ~~(SEQ. ID No.1)~~ (SEQ ID NO:1) with deduced amino acids ~~(SEQ. ID No. 2)~~ (SEQ ID NO:2) for the human lactoferrin protein and signal peptide sequence.

Please substitute the below amended paragraph for the paragraph on page 7, lines 1-2.

Fig. 14 is the (A) cDNA sequence ~~(SEQ. ID No. 3)~~ (SEQ ID NO:3) with (B) deduced amino acids ~~(SEQ. ID No. 4)~~ (SEQ ID NO:4) for the bovine lactoferrin protein.

Please substitute the below amended paragraph for the paragraph on page 7, lines 3-4.

Fig. 15 is the (A) cDNA sequence ~~(SEQ. ID No. 5)~~ (SEQ ID NO:5) with (B) deduced amino acids ~~(SEQ. ID No. 6)~~ (SEQ ID NO:6) for the porcine lactoferrin protein.

Please substitute the below amended paragraph for the paragraph on page 8, lines 11-21.

For the purposes of the present application, the term "substitution analog" referring to a DNA sequence means a DNA sequence in which one or more codons specifying one or more amino acids of lactoferrin or a lactoferrin polypeptide are replaced by alternate codons that specify the same amino acid sequence with a different DNA sequence. Where "substitution analog" refers to a protein or polypeptide it means the substitution of a small

number, generally five or less, commonly 3 or 4, and more often 1 or 2 amino acids as are known to occur in allelic variation in human and other mammalian proteins wherein the biological activity of the protein is maintained. For example, hLF isolated from milk has been reported to differ from the hLF of ~~SEQ. ID No. 2~~ SEQ ID NO:2 at two amino acid residues.

Please substitute the below amended paragraph for the paragraph on page 11, lines 5-14.

Fig. 2 is the cDNA sequence ~~(SEQ ID No. 1)~~ (SEQ ID NO:1) with the deduced amino acids ~~(SEQ ID No. 2)~~ (SEQ ID NO:2) for the secretion signal peptide and the mature human lactoferrin protein. The numbers on Fig. 2 correspond to the nucleotides starting at the 5' end. There are binding sites for two iron atoms with four amino acids participating in the binding of each iron. The amino acids at positions Asp80, Tyr112, Tyr209, and His273 are required for coordination with one iron, and amino acids at positions Asp415, Tyr455, Tyr548, and His617 bind the other. There are two glycosylation sites at position Asn157 and Asn498. The numbers refer to the deduced amino acid sequence. There are 25 amino acids per line of protein sequence (starting at nucleotide 18).

Please substitute the below amended paragraph for the paragraph on page 14, lines 19-29.

The invention also comprises partial sequences of the cDNA of ~~SEQ ID No. 1, 3 and 5~~ SEQ ID NO:1, 3 and 5 and substitution analogs thereof which code for biologically active polypeptides having homology with a portion of lactoferrin, especially those that are not available from enzyme digests of natural lactoferrins, the method of making polypeptides by use and expression of partial cDNA sequences, and the polypeptide products produced by the methods of this invention. The desired partial sequences can be produced by restriction enzyme cleavage, as for example at the cleavage sites indicated in Figures 18, 19 and 20. ~~the~~ The partial sequences may also be synthesized or obtained by a combination of cleavage, ligation and synthesis, or by other methods known to those skilled in the art.

Please substitute the below amended paragraph for the paragraph on page 19, lines 1-28 and page 20, lines 1-11.

EXAMPLE 3

PLASMID CONSTRUCTION

A schematic representation of the expression plasmid is shown in Fig. 5. The complete cDNA encoding human LF was repaired using the Klenow fragment of DNA polymerase I and subcloned into Acc I digested and repaired pGEM4 to generate pGEMhLFc. In order to remove the LF signal sequence and generate a 5' end in frame with the α-amylase sequences, a 252 base pair lactoferrin fragment (nt 69-321) containing Hind II/Acc I ends was obtained by polymerase chain reaction (PCR) amplification of pGEMhLFc plasmid DNA. The oligo primers used were as follows: the 5' end oligonucleotide as shown in ~~SEQ. ID. No.7~~ SEQ ID NO:7:

(CTGGGTCGACGTAGGAGAAGGAGTGTTCAGTGGTGC)

and the 3' end oligonucleotide as shown in ~~SEQ. ID. No. 8~~ SEQ ID NO:8:

(GCCGTAGACTTCCGCCGCTACAGG).

This PCR fragment was digested with Hind II and Acc I and was subcloned into Hind II/Acc I digested pGEMhLFc generating pGEMhLF. A 681 base pair α-amylase fragment with Asp718/Pvu II ends encoding the ~~promotor~~ promoter, signal sequence and the alanine residue from the start of the mature α-amylase II gene, was obtained by PCR amplification of *A. oryzae* genomic DNA. The oligo primers were as follows: the 5' end oligonucleotide as shown in ~~SEQ. ID. No. 9~~ SEQ ID NO:9:

(GAGGTACCGAATTCATGGTGTTTTGATCATTTTAAATTTTTATAT)

and the 3' end oligonucleotide as shown in ~~SEQ. ID. No. 10~~ SEQ ID NO:10:

(AGCAGCTGCAGCCAAAGCAGGTGCCGCGACCTGAAGGCCGTACAG).

The amplified DNA was digested with Asp 718 and Pvu II and subcloned into Asp718/Hind II digested pGEMhLF. The resulting plasmid (pGEMAhLF) was digested with EcoR I and the resulting 2.8 kb α-amylase-lactoferrin fragment was subcloned into a unique EcoR I site in pAL3 according to the method of generating pAhLF*. Synthetic oligonucleotides were used to provide the last five carboxy terminal codons of lactoferrin (nt 2138 – 2153) missing in pAhLF* and also to provide the first 180 bp of 3' untranslated sequences from the *A. niger* glucoamylase gene. The resulting plasmid ~~(pAhLFG)~~ (pAhLFG) was used to transform the *A. oryzae* pryG mutant strain.

Please substitute the below amended paragraph for the paragraph on page 25, lines 7-17.

EXAMPLE 9

DEGLYCOSYLATION

Deglycosylation was performed using N-glycosidase F (Boehringer Mannheim). *A. oryzae* growth medium containing 0.5 μg lactoferrin was denatured for 3 minutes at 100° C in the presence of 0.01% SDS. Standard LF from human milk was treated similarly. The samples were subsequently placed on ice for five minutes. N-glycosidase F reactions were conducted in 0.4 M sodium phosphate, (pH 6.8); 0.08% ~~Triton~~ TRITON®; 0.1% β-mercaptoethanol and 1 unit of enzyme and incubated at 37° C for sixteen hours. PAGE and Western analysis was performed using an IgG specifically directed against human lactoferrin to detect an increase in mobility of digested samples.

Please substitute the below amended paragraph for the paragraph on page 28, lines 11-30.

Western Immunoblot analysis was performed to determine if the 3' iron binding domain was expressed in the bacterial cells under the control of the T7 promoter and to monitor its purification. The cells were harvested at 5000*g* and resuspended in 15 ml of PBS (pH 7.4). Total cellular extracts were prepared by sonication for 1 minute on ice. The

sonicate was centrifuged at 13,000*g* for 50 minutes at 4° C. The supernatant was removed and the pellet was resuspended in 50 ml of denaturation buffer (5M urea, 2% ~~triton~~ TRITON®, 5mM EDTA, 0.01% Tween 20, 50 mM TrisCl, pH 7.5) and centrifuged at 48,000*g* for one hour. The supernatant containing the soluble fraction was recovered. Protein concentration was determined using the Bradford reagent according to manufacturers instructions (BioRad, Richmond, CA). Protein samples (40 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose filter electrophorectically using the Western Immunoblot procedure. The filter was blocked with Tris-buffered saline (TBS, 0.05 M Tris/0.15 M NaCl, pH 7.5) containing 2% dried milk, and then incubated for 2 hours in the same with the addition of a specific polyclonal IgG (1 μg/ml) directed against hLF (Sigma, St. Louis, MO). The filter was washed (5 x 10 min) in TBS/0.05% Nonidet P-40 followed by incubation with 5 μCi of ^{125}I protein A in TBS/2% dried milk. The filter was washed (5 x 10 min) in TBS/0.05% Nonidet P-40, dried and exposed overnight in Kodak XAR5 film at - 70°C. The film was developed by autoradiography.

Please substitute the below amended paragraph for the paragraph on page 30, lines 5-12.

A 156 bp human lactoferrin fragment encoding AA 1-52, containing Sma I/BamH I ends was obtained by polymerase chain reaction (PCR) amplification of pGEMhLFc plasmid DNA [Ward, P. P., *et al., Biotechnology*, **10**:784-789 (1992)]. The oligonucleotide primers used were as follows:

5' end oligonucleotide as shown in ~~SEQ. ID. NO. 11~~ SEQ ID NO:11

CTGCCCGGGCGTAGGAGAAGGAGTGTT

3' end oligonucleotide as shown in ~~SEQ. ID. No. 12~~ SEQ ID NO:12

CATGGATCCTGTTTTACGCAATGGCCTGGATACA

Please substitute the below amended paragraph for the paragraph on page 30, lines 21-30 and page 31, lines 1-8.

pGEX-3XLFN-1 was transformed into the bacterial strain, JM109. Transformants obtained were cultured overnight in LB (50 ml) containing ampicillin (50g/ml) at 37° C/250 rpm. Overnight cultures were subcultured into LB (500ml) containing ampicillin (50g/ml) and grown at 37° C/250 rpm until an $0D_{600}$nm of 0.6-0.8 was obtained. Isopropyl-D-thiogalactopyranoside (IPTG) was added to the culture medium at a concentration of 1 mM to turn on the *tac* promoter resulting in expression of the glutathione S-transferase/LFN-1 fusion protein. Growth under these conditions continued for 4 hours after which the cells were harvested at 5,000g and resuspended in 5 ml of MTPBS (150mM NaCl, 16mM Na_2HPO_4, 4mM NaH_2PO_4, 1% ~~Triton~~ TRITON® X-100, pH 7.3). Total cellular extracts were prepared by 3 X 1 minute freeze/thaw cycles followed by mild sonication for 2 X 1 minute. The sonicate was centrifuged at 13,000g for 20 minutes and the supernatant obtained was applied to a glutathione sepharose 4B column following manufacturer's instructions (Pharmacia, Piscataway, N.J.). The glutathione S-transferase/LFN-1 fusion protein was eluted from the column using 10 ml of elution buffer (10 mM glutathione, 50 mM Tris pH 8.0). Fractions of 1.5 ml were collected and dialyzed overnight against 50 mM Tris, 15% glycerol pH 8.0.

Please substitute the below amended paragraph for the paragraph on page 32, lines 1-21.

EXAMPLE 13

EXPRESSION OF BOVINE AND PORCINE LACTOFERRIN IN *ASPERGILLUS ORYZAE*.

A universal *A. Oryzae* expression vector is constructed to allow in frame subcloning of any cloned cDNA of interest. This vector, pAG, is similar to the vector pAhLFG(+1) utilized for the expression of human lactoferrin in *A. Oryzae* above. A 680 bp α-amylase fragment encoding the promoter, signal sequence and the alanine residue from the start of the mature –amylase II gene, is obtained by polymerase chain reaction (PCR) amplification of pAhLFG(+1).

The oligonucleotide primers are as follows:

5' end oligonucleotide, ~~SEQ. ID. NO. 13~~ SEQ ID NO:13

5' CGGAATTCATGGTGTTTTGATCATTTT

3' end oligonucleotide, ~~SEQ. ID. NO. 14~~ SEQ ID NO:14

5'TGGAATTCGATCGCGGATCCGCAATGCATGCAGCCAAAGCAGGTGCCGC GAC

The 5' end oligonucleotide encodes an EcoR I site and the 3' end oligonucleotide contains an Nsi I site, flanked by a BamH I site. This amplified DNA is digested with EcoR I and BamH I and subcloned into EcoR I/BamH I digested pAhLFG(+1) generating pAG. All PCR amplified products and construction junctions are sequenced using the commercially available Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH).

Please substitute the below amended paragraph for the paragraph on page 33, lines 5-15.

EXAMPLE 14

EXPRESSION OF HUMAN LACTOFERRIN IN *SACCHAROMYCES CEREVISIAE*

The complete human lactoferrin (hLF) cDNA was expressed in *Saccharomyces cerevisiae* using the yeast expression plasmid, YEP [McDonnell, D.P. *et al., J. Steroid Biochem, Molec. Biol.*, **39**:291-297 (1991)]. A 2.2kb fragment encoding the complete hLF cDNA ~~SEQ. ID No. 1~~ SEQ ID NO:1 was generated using the polymerase chain reaction. This fragment contained ~~and~~ an *XhoI* restriction enzyme site at its 5' end and an *Asp718* restriction enzyme site at its 3' end. The 2.2kb fragment was subcloned, in frame, into *XhoI/Asp718* digested YEP to yield, YEPLFc.

Please substitute the below amended paragraph for the paragraph on page 35, lines 1-18.

EXAMPLE 15

EXPRESSION OF hLF IN *ASPERGILLIS NIDULANS*.

Construction of the *Aspergillis Nidulans* Expression Plasmid.

The plasmid used for expression of *hLF* cDNA is shown schematically in Fig. 17. The cDNA of ~~SEQ. ID No. 1~~ SEQ ID NO:1 as a 2.3-kb clone contained the secretory signal sequence and complete translation frame. The sequence of the entire cDNA was confirmed by dideoxy sequence analysis (Sequenase version 2.0, U.S. Biochemical, Cleveland, OH). The cDNA was repaired using the PolIk and subcloned into *Acc*I-digested and blunt-ended pGEM4. The plasmid, pGEMhLF, was digested with *Hind*III + *Asp*718 and repaired using PolIk. The resulting 2.3-kb *hLF* fragment was subcloned into a unique *Sma*I site located in the multiple cloning cassette of pAL3 downstream from the *alc*A promoter, Waring, R.B., *et al., Gene*, **79**, 119-130 (1989), generating pAL3hLF. The β-tubulin transcription terminator fragment was obtained by digesting the 3'-untranslated region of the *benA* gene (nt 2569-2665; ~~May et al.~~ *May et al.*, 1987) with *XbaI* + *NheI* and subcloned into *XbaI*-digested pAL3hLF generating pAL3hLFT. This plasmid was used to transform *A. nidulans* strain GR5 (*pyrG89; wa3; pyroA4*).

Please substitute the below amended paragraph for the paragraph on page 40, lines 12-23 and page 41, lines 1-17.

EXAMPLE 16

PRODUCTION OF DNA SEQUENCE SUBSTITUTION ANALOGS.

Figure 18 shows the restriction enzyme cleavage sites in the ~~SEQ I. D. No. 1~~ SEQ ID NO:1 cDNA for cleavage by various endonucleases. Table 2 lists the alternative codons that code for the 20 common amino acids. DNA sequence substitution analogs that also code for human lactoferrin can be constructed by choosing alternate codons from Table 2 to alter the

DNA Sequence between a pair of cleavage sites selected from Fig. 18. Alternative codons are assembled into a synthetic oligonucleotide by conventional methods and the synthetic oligo is substituted into the endonuclease treated DNA of ~~Sequence ID. No. 1~~ SEQ ID NO:1 by the methods described in "Molecular Cloning. A Laboratory Manual", 2d Edition, Cold Spring Harbor Laboratory Press (1989), to produce a substitution analog. Other methods generally known to those skilled in the art can also be employed to obtain substitution analogs of DNA sequences. The alteration of the DNA by cleavage and codon substitution ~~maybe~~ may be repeated to substitute substantial portions of the original DNA sequence with alternative codons without altering the protein expressed by the DNA of ~~Sequence ID. No. 1~~ SEQ ID NO:1. The same methods can of course be used to make substitution analogs of the cDNA of ~~SEQ ID No. 3 and 5~~ SEQ ID NO:3 and 5. Alteration of a DNA sequence which produces no change in the protein expressed by the DNA sequence might, for example, be conducted to increase protein expression in a particular host cell by increasing the occurrence of codons that correspond to amino acid tRNAs found in higher concentration in the host cell. Such altered DNA sequences for substitution analogs can be easily produced by those of ordinary skill in the art following the method set out above, or other alternative techniques for altering the DNA sequence while obtaining the same protein on expression. Substitution analogs can be obtained by substitution of oligonucleotides at restriction cleavage sites as described above, or by other equivalent methods that change the codons while preserving the amino acid sequence of the expressed protein.